CHAPTER II

MATERIALS AND METHODS

1. Bacterial Strains

Ninety-nine clinical isolates of *Neisseria gonorrhoeae* were studied in the experiments. Twenty-eight were freshly isolated from patients attending V.D. clinic, Rajdamri, Bangkok, and 71 isolates were kindly provided as subcultures from Bangrak Hospital, Bangkok.

Other bacterial strains including six strains of *N. meningitidis*, four of *N.sicca*, three of *N.mucosa*, and a single strain of *Moraxella osloensis* were kindly provided by Assistant Professor Chatchai Sornchai, Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University. One strain each of *Branhamella catarrhalis*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* were from clinical isolate from the Department of Microbiology, Faculty of Medicine, Chulalongkorn University.

All isolates were kept in 10% skim milk at-70°C until study.

2. Laboratory Animal

Young New Zealand White rabbits , male and female

weighing about 2 kg were obtained from Laboratory Animal Center, Faculty of Medicine, Chulalongkorn University.

3. Culture of N.gonorrhoeae (3,7,15)

Clinical specimens and subculture of gonococci were inoculated on Thayer-Martin and chocolate agar media. The cultures were inspected after 24 and 48 hours incubation at 36 - 37°C in a humid atmosphere in candle jar. The presumptive identification of N.gonorrhoeae was based on typical colony morphology, a positive oxidase reaction and gram negative diplococci on microscopic examination. All presumptively positive isolates were confirmed by Phadebact coagglutination test (Pharmacia).

4. Colony typing of N.gonorrhoeae

Colonial types were classified by the technique and criteria described by Kellogg et al. (55), and Jephcott and Reyn (123) as viewed through a stereomicroscope using diffuse, obliquely transmitted light after the isolated gonococcal colonies were grown on GCBS in candle jar at 37°C for 18-24 hours. Distinguishing features were as follows:

Type 1 : Small (0.5 mm in diameter), dark gold coloration with entire edge, convex and highly light reflecting.

Type 2 : Small (0.5 mm in diameter), dark gold colour, convex, defined or crenated edge with highly light reflecting around the edge. and friable consistency.

Type 3 : Larger (1 mm in diameter) and flatter than types 1 or 2, round, light brown granular appearance, and entire edge.

Type 4 : Similar in size and shape to type 3, but amorphous and colourless.

Type 5 : Similar in size to type 3 or 4, dark brown in coloration with coarsely irregular edge, granular and frequently a series of concentric rings on the surface of the colony.

5. Electron Microscopy (160)

Whole gonococcal cells or other bacteria were visualized by electron microscope for the presence or absence of surface pili. Colonies of each microorganism were scraped with a platinum loop and dispersed in a drop of fixative solution. A carbon-collodion coated copper grid (300 mesh) was inverted and floated on the surface of a drop of each of the bacterial suspension. After 3-5 min, the excess fluid was removed by touching with the torn edge of Whatman No. 1 filter paper, then transfered to float on a drop of the phosphotungstate solution, 0.5% for 3 min. The solution was

removed and the grid was dried at room temperature in desicator. The grids were examined with a JEOL, JEM 200 CX transmission electron microscope (JEOL Instruments, Japan) operating at 80 KV.

Purified gonococcal pili were examined as above without fixative.

6. Protein Determination

Estimation of protein concentration was performed by the method of Lowry et al. (161).

7. Immunodiffusion

Double immunodiffusion was carried out in 1% agarose in saline as previously described (66).

8. Purification of Gonococcal Pili

Gonococci used in purification was freshly isolated from patients with gonococcal infection, the colony type was determined and the presence of pili confirmed by electron microscopy. Purification of gonococcal pili was performed according to the method of Hermodson et al. (117).

Piliated gonococci were inoculated onto 200 Kellogg

medium plates (9 cm in diameter). After 18-24 hours incubation at 37°C in candle jar, the bacteria were harvested into 100 ml of ice-cold 0.01 M Tris-HCl,pH 9.5. Bacterial suspension was then sheared for 2 min at top speed in a Sorvall Omnimixer (Ivan Sorvall Inc., New town, Conn., USA.) and the bacteria, debris removed from the suspension by centrifugation at 12,000 xg 4°C for 10 min (J2 - 21 centrifuge, Beckman Instruments., USA.). The supernate was then centrifuged at 48,000xg for 60 min, 4°C of which the supernate was mixed well with an equal volume of 20% saturated ammonium sulfate and stirred at 4°C overnight. The ammonium sulfate suspension was centrifuged at 30,000 xg for 10 min after which the pellet was redissolved and precipitation with ammonium sulfate was then repeated. The pili pellet was finally suspended in 0.5 ml of 0.01 M Tris-HCl pH 7.0 containing 0.01 M NaN_3 and protein determination was performed by the method of Lowry et al.

9. Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate (SDS-PAGE)

SDS-PAGE was performed in 0.6 mm slab gels by the method of Laemmli (162) with 12.5% running gels as described previously (112).

For sample preparation, 4 volumes of purified gonococcal pili suspension was mixed to one volume of stock

sample buffer, boiled in the waterbath for 5 min and allowed to cool, Bromphenol blue was included in the buffer to serve as tracking dye.

The separating gel solution was prepared as described in Table 4, poured into gel mould as shown in Fig. 9, overlaid with distilled water and left to polymerize completely. The polymerization was accomplished when a sharp interface was visible between the gel and the aqueous layers. Water from the separating gel surface was then rinsed off and the stacking gel mixture (as prepared in Table 4), was filled in the remaining space in the mould. A comb was inserted into the top of the gel, ensuring that no air bubbles were trapped and left to polymerize completely for about one hour at room temperature. The gel mould containing the polymerized gel was clamped to the electrophoresis tank(Fig.9,10) and electrode reservoirs were filled with electrode buffer.

Fifteen to twenty microlitre of each treated samples and 10 ul of standard molecular weight markers were loaded on the gel and electrophoresed at 15 mA until the bromphenol blue tracking dye reached the bottom of the separating gel.

The gel was stained in the staining solution overnight at room temperature , removed from staining solution and rinsed quickly in water and placed in destain

solution I. Destain solution were frequently shaked and changed until a clear background was obtained. The gel was stored in destain solution II.

9.1. Molecular Weight Estimation

The migratory distance of tracking dye and of blue protein zones from the top of gel were recorded to determine the relative mobility (R_F) of the protein as follows:

$$R_F = \frac{\text{distance of protein migration}}{\text{distance of tracking dye migration}}$$

The R_F values of standard protein markers were plotted against the known molecular weights of the corresponding markers on semi-logarithmic paper, and the molecular weight of unknown protein was estimated from the calibration curve.

10. Preparation of Rabbit Anti-Gonococcal Pili Antibody

Anti-pili antibody was raised in rabbit according to the method of Buchanan and Pearce (65), fifty micrograms of purified gonococcal pili protein in 0.01 M Tris-HCl,pH 7.0 were emulsified in equal volume of Freund's complete adjuvant using two syringes technique until a stable water in oil emulsion was obtained. This was verified by gently

placing one drop from syringe onto surface of water; the drop would not dispersed when the emulsion was stable. The antigen emulsion was injected intramuscularly into both hind limbs of the rabbits. Three weeks later the rabbit received a booster dose of the same concentration of pili emulsified in an equal volume of Freund's incomplete adjuvant and the rabbit was test bled seven days after the last injection. The serum was separated and antibody titre determined by immunodiffusion and IHA. Antisera with high titered were stored at - 70°C until use.

11. Indirect Haemagglutination Test (IHA) (39)

11.1. Formalin Treatment of Sheep Red Blood Cells

SRBC were washed 6 times in NSS and then diluted to 1:10 in NSS. The erythrocyte suspension was treated with an equal volume of 7.5% formalin and incubated in a shaker waterbath at 37°C for 18-20 hours. The SRBC were washed four times with NSS and finally resuspended in NSS as a 10 percent suspension.

11.2. Tannic Acid Treatment

The formalin treated SRBC were washed once in NSS , resuspended as a 2% suspension in PBS pH7.2 and mixed with equal volume of tannin solution. The mixture was

incubated in a waterbath at 37°C for 30 min, the SRBC were centrifuged down, washed twice in PBS pH 6.4,and resuspended as a 2% suspension in the same buffer. This suspension could be used up to 6 weeks when stored at 4°C.

11.3. Sensitization

Pili antigen diluted to 1:1000 (This optimal dilution was kindly performed by Dr.Karin Reiman, Statens Seruminstitut, Copenhagen, Denmark) in PBS pH 6.4, was sonicated for 60 seconds at 9 micron with an MSE soniprep150 ultrasonic disintegrator (MSE Scientific Instruments) immediately before use. The diluted pili antigen was mixed with an equal volume of 2% suspension of tanned formalin treated SRBC in PBS pH 6.4 and incubated 1 hour in a 37°C waterbath. The sensitized SRBC were then washed twice in 1% PBS-R and resuspended in 1% PBS-R as a 1% suspension.

SRBC treated in the same way with PBS pH 6.4, without antigen, were used as control cells.

11.4. Performance of the Test.

Rabbit antiserum was inactivated at 56°C for 30 min and diluted to 1:10 with PBS pH 6.4. The titration of antiserum was performed in a microtitration plate (U-shaped wells, Flow Laboratories). Two-fold serial dilutions from

initial 1:10 were made in 1% PBS-R, the volume in each well being 25 ul. 25 ul of a 1% suspension of sensitized SRBC was added to each well. The serum sample and PBS alone were also tested with 1% control cell and sensitized cell respectively. The positive and negative sera control were also included in the experiment. The plate was carefully shaken, covered and was left overnight at room temperature before reading. The titre was recordeded as the reciprocal value of the lowest serum concentration giving 1^{+} reaction.

12. Coagglutination Test

The stabilization and coating of protein A-containing staphylococci to make coagglutinating reagent followed procedures described by Johnston (163).

12.1. Preparation of Stabilized Staphylococcal Protein A.

Staphylococcus aureus, strain Cowan I was grown overnight in TSB at 37°C. The harvested bacteria were washed three times in PBS pH 7.2 and treated for 3 hours with 0.5% formalin , washed twice in PBS, resuspended,and exposed to 80°C for 5 min. After additional washing, the bacteria were suspended to 10% in PBS containing 0.1% NaN_3 and kept at 4°C until use.

12.2. Sensitization

One ml of the 10% suspension of protein A-containing staphylococci was added to 0.1 ml of rabbit antiserum. After the suspension was mixed and left for 10 min at room temperature, the staphylococci were washed twice in PBS and suspended to 1% in PBS containing 0.1% NaN_3.

Control reagent consisted of staphylococci coated with normal rabbit serum in place of the antiserum.

The suspensions were kept at 4°C.

12.3. Test Procedure (163)

Two smears of bacterial growth were made on a microscopic slide with a calibrate platinum loop (0.001 ml). One drop of the test reagent was added to one smear and control reagent to the other. The drops were mixed thoroughly into the smears with a platinum loop or applicator. The slide was then rocked gently for 2-3 min and observed for agglutination with naked eye under oblique light against a dark background. Test was read as follows:

Positive : definite clumping of coarse or fine particles with sensitized reagent within 3 min, the control remaining evenly turbid.

Negative : both suspensions evenly turbid with no visible clumping.

13. Isolation of IgG from Rabbit Antiserum

The IgG from rabbit antiserum to purified gonococcal pili was fractionated by DEAE chromatography at pH 8.0 (164).

Ten grams wet weight of DE 52 were added to 100 ml of starting buffer. The slurry was gently stirred, the pH brought to 8.0, and allowed to settle. The supernatant fluid was removed by suction and resin washed in the same buffer. The pH rechecked and adjusted when necessary. The bottom outlet of the column was closed and filled to 10 cm height with starting buffer. The slurry was swirled and poured into the column, allowed to settle under gravity for about 1 hour. The botton of the column was opened and the resin allowed to pack. The column was washed with starting buffer until the pH of the eluent was exactly the same as the starting buffer.

Four ml of rabbit antiserum was dialysed overnight against 1 litre of the starting buffer, and insoluble material removed by centrifugation. The serum was applied to the column and then eluted with starting buffer at a flow rate of 30 ml/hr. Three ml fractions were collected and the absorbance monitored at 280 nm. IgG was eluted by the

starting buffer in the first asymmetric peak as shown in Fig.21.

14. Immunoelectrophoresis (IEP)

IEP was performed in 1.5% agarose in barbital buffer according to the method previously described (164).

15. Preparation of Alkaline Phosphatase Conjugated Rabbit Anti-gonococcal Pili IgG

The conjugation of alkaline phosphatase to rabbit anti-pili IgG antibody was performed by one-step glutaraldehyde method of Avrameas (165).

A mixture of rabbit IgG (0.5 mg) and 1.5 mg of alkaline phosphatase enzyme , was dialysed overnight against PBS-Mg^{2+} at 4°C. Glutaraldehyde (10%) was added dropwise to give a final concentration of 0.2% while the mixture was gently stirred. After the reaction mixture was allowed to stand for 3 hours at room temperature , 0.1 ml of 1 M lysine pH 7.0 was added and the mixture dialysed at 4°C overnight against PBS-Mg^{2+}. The precipitate formed was removed by centrifugation for 20 min at 40,000 xg and the supernate was added with 0.1 ml of 20 mg/ml BSA and with 4 ml of 0.05 M Tris-HCl pH 8.0. The conjugate was filtered through a sterile millipore membrane (0.22 um) and maintained in the dark at 4°C until use.

16. Determination of Working Strength of Antipili Conjugate and of Antipili IgG Coating Level (166)

The optimal concentration and dilution of rabbit antipili IgG and of rabbit antipili IgG conjugate used in our ELISA system for detection of pili antigen were determined by checkerboard titration as folowing:

Rabbit IgG of the antiserum against purified pili was diluted in coating buffer to give 10,5,1, and 0.1 ug/ml; 100 ul of each dilution was added to duplicate horizontal rows of wells in a microplate (Nunc) and the plate was covered and incubated overnight at 4°C in a humid chamber. The plate was washed three times with PBS-T. 100 ul of 5 ug/ml sonicated gonococcal pili in sample diluent were added to each vertical column of wells of each antibody conjugate dilutions and incubated the plate at 37°C for 3 hours. After the plate was washed four times with PBS-T , 100 ul of each conjugate dilutions, diluted with enzyme diluent to 1:200, 1:400, 1:500, 1:600, 1:700, 1:800, were added to duplicate vertical columns of wells (Fig 11). The plate was incubated at 37°C for 3 hours and washed four times with PBS-T. The 100 ul of substrate was added to each wells and the incubation was allowed to proceed at 37°C in a humid box. After 30, 45 and 60 min of enzyme-substrate reaction, the absorbances of all wells of the plate were read at 405 nm with a Titertek Multiskan (Flow Laboratories. West Germany).

17. Determination of Optimal Antigen and Conjugate Incubation Time

17.1. Incubation of Pili Antigen with Rabbit Anti pili IgG Coated Plate

The optimal antigen binding was determined by doing titration with varying antigen incubation period at 37°C for 30, 45, 60, and 120 min as follows: Each well of plates was coated with 100 ul of antipili IgG at an optimal antibody coating level(from checkerboard titration above) and then incubated at 4°C overnight. After the antibody was removed, the plates were washed 3 times with PBS-T. One hundred microlitres of pili antigen was added in each well, and the plates were incutated at 37°C for 30, 45, 60, and 120 min. After incubation at various times, the plates were washed four times, then 100 ul of suitably diluted conjugate (chosen from checkerboard titration above) was added. The plates were incubated at 37°C for 120 min. The excess conjugate was washed out 4 times with PBS-T, and then 100 ul of substrate was added. After 60 min at 37°C, the absorbance of the colour developed was read at 405 mm with a Titertek Multiskan.

17.2. Incubation with Conjugate

The duration for conjugate incubation was

determined in a similar manner by varying incubation period of conjugate at 37°C, ranging from 30 to 180 min.

18. Double Antibody Sandwich ELISA for Detection of Gonococcal Pili Antigen

18.1. Preparation of Sample

Gonococcal pili suspension was diluted to a appropriate concentration with sample diluent, and was then sonicated at 9 micron for 60 seconds. Whole bacteria were suspended in sample diluent and adjusted to an optical density of 0.3 at 540 nm that corresponded to 10^8 cell/ml. When appropriate, the bacterial suspensions were sonicated at 10 micron for 2-5 min before use.

18.2. Performance of the Assay

The rabbit anti-pili IgG antibody was diluted with coating buffer to a protein concentration of 5 ug/ml (from checkerboard titration). One hundred microlitres of antibody was added to each well of microplate and the plate was then incubated at 4°C overnight. After the antibody was removed,the plate was washed 3 times with PBS-T. 100 ul of samples were added to the wells in duplicate. The plate was incubated at 37°C for 45 min , washed four times with PBS-T and 100 ul of diluted conjugate (1:300) was added.

After incubation at 37°C for 45 min, the plate was again washed 4 times,and 100 ul of substrate was added. The enzyme substrate reaction was allowed to proceed for 60 min at 37°C before being terminated by the addition of 50 ul of stopping solution. The absorbances of the wells were read spectrophotometrically at 405 mm.